Efficient Biooxidations Catalyzed by a New Generation of Self-Sufficient Baeyer-Villiger Monooxygenases

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Contents

Overexpression of CRE2/BVMOs

Expression of the new generation of self-sufficient BVMOs in *Escherichia coli* TOP10 yielded up to 50 mg of pure and soluble CRE2/BVMO from 1 L culture broth. In comparison to the previous generation of self-sufficient BVMOs, similar protein expression levels were obtained (Figure S1).

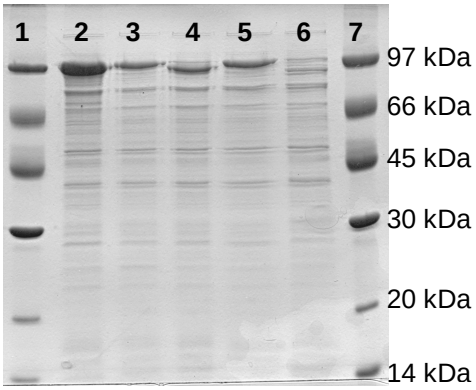

Figure S1. Soluble overexpression of CRE2/BVMOs in cell-free extract (CFE) of *E. coli* TOP10, visualized by a 12 % SDS-PAGE gel. *Lane 1 and 7* LMW marker (BioRad), *lane 2* CRE2−PAMO, *lane 3* CRE2−CHMO, *lane 4* CRE2−CPMO, *lane 5* CRE2−HAPMO, *lane 6* CRE2−EtaA.

Cloning Strategy pCRE2−PAMO linker variants

For the creation of pCRE2−PAMO linker variants 1 and 2, the original linker was removed using XhoI and PvuII restricition sites. The linearized vector was subsequently treated with Antarctica Phosphatase (New England Biolabs) according to the manufacturer's guidelines. The linker fragments were created using 5'-phosphorylated complementary primers (Table S1). These primers were dimerized in a thermocycler by incubating 100 μM of each primer in 10 mM Tris/HCl (pH 8.5) at 72 ºC for 2 min, followed by a slow cooling down to 23 ℃ in 45 min. The resulting double-stranded DNA was then ligated to the linearized vector using T4 DNA ligase (New England Biolabs).

Table S1. Linker fragments used to prepare pCRE2−PAMO linker variants 1 and 2.

Linker variant	**Nucleotide sequence (5' → 3')**
Variant 1 (glycine-rich)	P-tcgagtggtggctctggtgggagcggtggctcag
	P-ctgagccaccgctcccaccagagccaccac
Variant 2 (random, "nature-preferred")	P-tcgagtgccaccggtagcgcgacgggctcag
	P-ctgagcccgtcgcgctaccggtgccac

Linker variant 3 was created by amplifying the PTDH mutant gene and ligating in this amplified product in the NdeI/PvuII restricted pCRE2−PAMO vector. Prior to ligation, the PCR product was digested with NdeI. Subsequently, one nucleotide from the linker sequence was removed using the QuikChange® Site-directed Mutagenesis Kit from Stratagene. Primers used for both amplification and mutagenesis are shown in Table S2.

Table S2 Forward and reverse primers for PCR amplification and subsequent site-directed mutagenesis of the PTDH mutant gene.

Variant 3 (short)	**Nucleotide sequence (5' → 3')**
PCR forward primer	ggcagccatatgctgccg
PCR reverse primer	tcagccgcagggttggc
QuikChange forward primer	gccaaccctgcggctgactggatggccgggcagacg
QuikChange reverse primer	cgtctgcccggccatccagtcagccgcagggttggc

Overexpression and fusion enzyme purification

All CRE2−PAMO linker variants were overexpressed in *E. coli* TOP10 using Terrific Broth (TB) medium containing 50 µg mL^{-1} ampicillin and 0.02 % (*w/v*) L-arabinose. The CRE2−PAMO linker variants were overexpressed at 24 ℃ during 36 h. Thereafter, cells were harvested, washed and resuspended in 50 mM Tris/HCl, pH 7.5 containing 10 µM FAD. After breaking the cells by ultrasonication and removing cell debris by centrifugation, fusion enzymes were purified using 2 mL Ni-NTA column. After purification, excess of imidazole was removed using an Econo-PAC 10DG desalting column (BioRad). The fusion enzymes were concentrated by ultrafiltration and stored at −80 ºC in their resuspension buffers.

Thermostability measurements of CRE2−PAMO linker variants

Thermostability of the enzymes was determined by incubating approximately 10 μM of enzyme in the presence of 10 μM of FAD at 55 ℃, after which their remaining activity was determined at 25 ℃ using saturating sub strate concentrations. The CRE2−PAMO linker variants contained one of the following peptide sequences as linker:

Table 3 Amino acid sequence of the different CRE2-PAMO linker variants.

Linker variant	Peptide sequence
Normal	SRSAAG
Variant 1 (glycine-rich)	SSGGSGGSGGSAG
Variant 2 (random, “nature-preferred”)	SSATGSATGSAG
Variant 3 (short)	W

The remaining activity of both subunits of the CRE2−PAMO linker variants was followed in time:

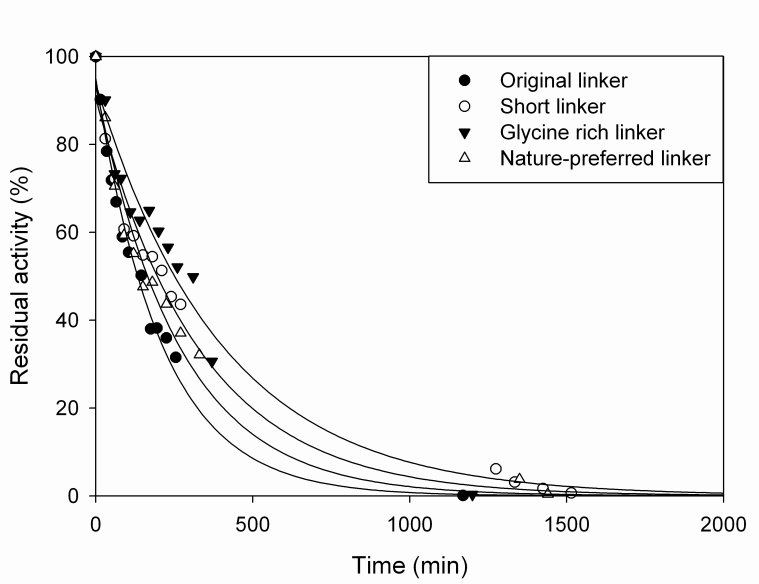

Figure S1. The residual activity of the PAMO subunit of CRE2−PAMO containing different linker variants at 55 ℃. The following life-times ($t_{½,\ 55\ ^oC}$) were determined for these variants; a) original linker, 208 min^{-1}; b) short linker, 333 min^{-1}; c) glycine-rich linker, 400 min^{-1}; d) nature-preferred linker, 264 min^{-1}.

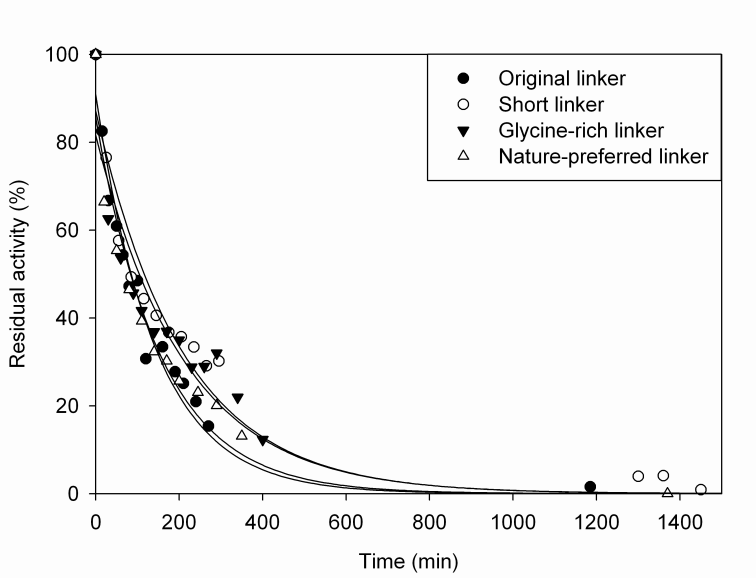

Figure S2. The residual activity of the PTDH subunit of CRE2−PAMO containing different linker variants at 55 ℃. The following life-times ($t_{½,\ 55\ ^oC}$) were determined for these variants; a) original linker, 142 min^{-1}; b) short linker, 208 min^{-1}; c) glycine-rich linker, 212 min^{-1}; d) nature-preferred linker, 156 min^{-1}.